IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTACH TO #4

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver Serial No.: 09/227,595 Continuation of Filed: ~~January 8, 1999~~ December 20, 2001 For: CTLA4-Cγ4 Fusion Proteins (as amended) Attorney Docket No.: RPN-001	Group Art Unit: 1642 Examiner: Helms, L.

MCW

Assistant Commissioner for Patents
Washington, D.C. 20231

MCW

Certificate ~~of First Class Mailing~~ (37 CFR ~~1.8(a)~~ under 1.10)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ "Express Mail to Addressee" in an envelope addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor
~~Megan E. Williams~~ Larry Taylor
~~Registration No. 43,270~~
~~Attorney for Applicants~~

Mailing Label No. EL 833159141US

MCW

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT

AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

2. Prior to July 11, 1994, the invention described and claimed in the above-referenced application was completed in this country, as evidenced by the following:

A. Prior to July 11, 1994, we were in possession of a cloned CTLA4Ig molecule which was producing large amounts of active protein in CHO DG44 cells.

Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

Page A-2 of Appendix A describes amplification of the mutated γ4 Hinge-CH_2-CH_3 region and the cloning of the mutated γ4 into pNRDSH/hCTLA4 to replace the existing γ1 Hinge-CH_2-CH_3.

Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: Oct. 4, 2001 Signed: Gary S Gray

Gary S. Gray

Date: _____ Signed: _____

Jerry Carson

Date: _____ Signed: _____

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: _____ Signed: _____

Sandra Silver

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray et al. Serial No.: 08/595,590 Filed: February 2, 1996 For: CTLA4-Immunoglobulin Fusion Proteins Having Modified Effector Functions and Uses Therefor Attorney Docket No.: RPI-007	Group Art Unit: 1806 Examiner: Eyler, Y.

Assistant Commissioner for Patents
Washington, D.C. 20231

Certificate of First Class Mailing (37 CFR 1.8(a))

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2-20-98
Date of Signature and of Mail Deposit

By: Giulio A. DeConti
Giulio A. DeConti, Jr.
Reg. No. 31,503

ASSOCIATE POWER OF ATTORNEY

Sir:

The undersigned attorney has the power of attorney in the subject application. He hereby grants an associate power to:

Megan E. Williams, Ph.D.
Registration No. P43,270
Lahive & Cockfield, LLP
28 State Street
Boston, MA 02109

Please continue to forward all written and telephonic communications to Amy E. Mandragouras at the address and telephone number listed below.

Respectfully submitted,

Giulio A. DeConti, Jr.
Registration No. 31,503
Attorney for Applicants

LAHIVE & COCKFIELD, LLP
28 State Street
Boston, MA 02109
Tel. (617) 742-4214

Dated: February 20, 1998

human - CD4 : Ig

STATUS : This clone, which is currently producing large amounts of active protein in CHODG44, is being methotrexate amplified.

RECENTLY focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 4..
Tuno et al, 1991 J Immunol. (147)
TAO et al., 1991 J Exp Med (173) 02..
Duncan + Winter, 1988 NATURE (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH2 domain from γ1 and mutated residue 235 and 237 in γ4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me Date

Scott R Carr

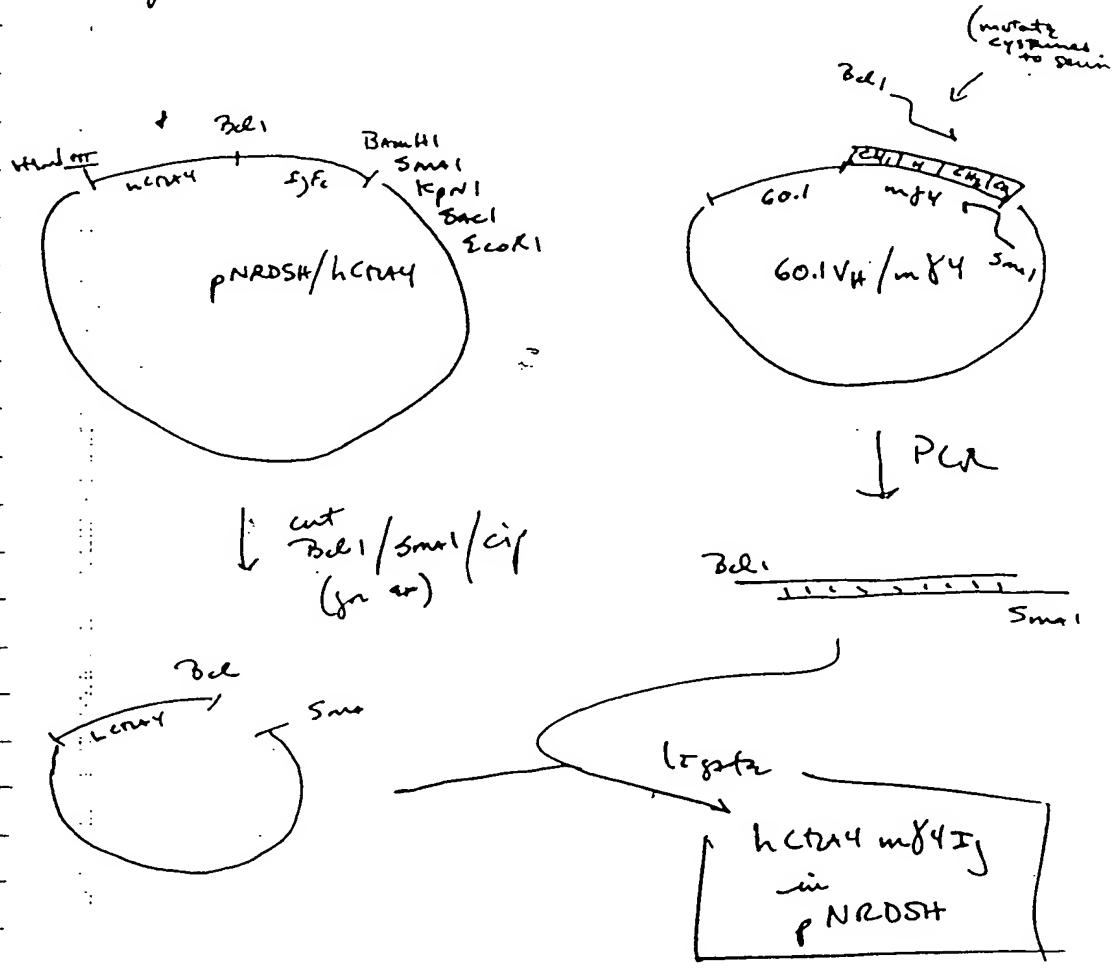
2 STRATEGIES will BE USED:

hCγ4, mutagenesis of IgFc

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the EXISTING γ_1 H-CH_2-CH_3

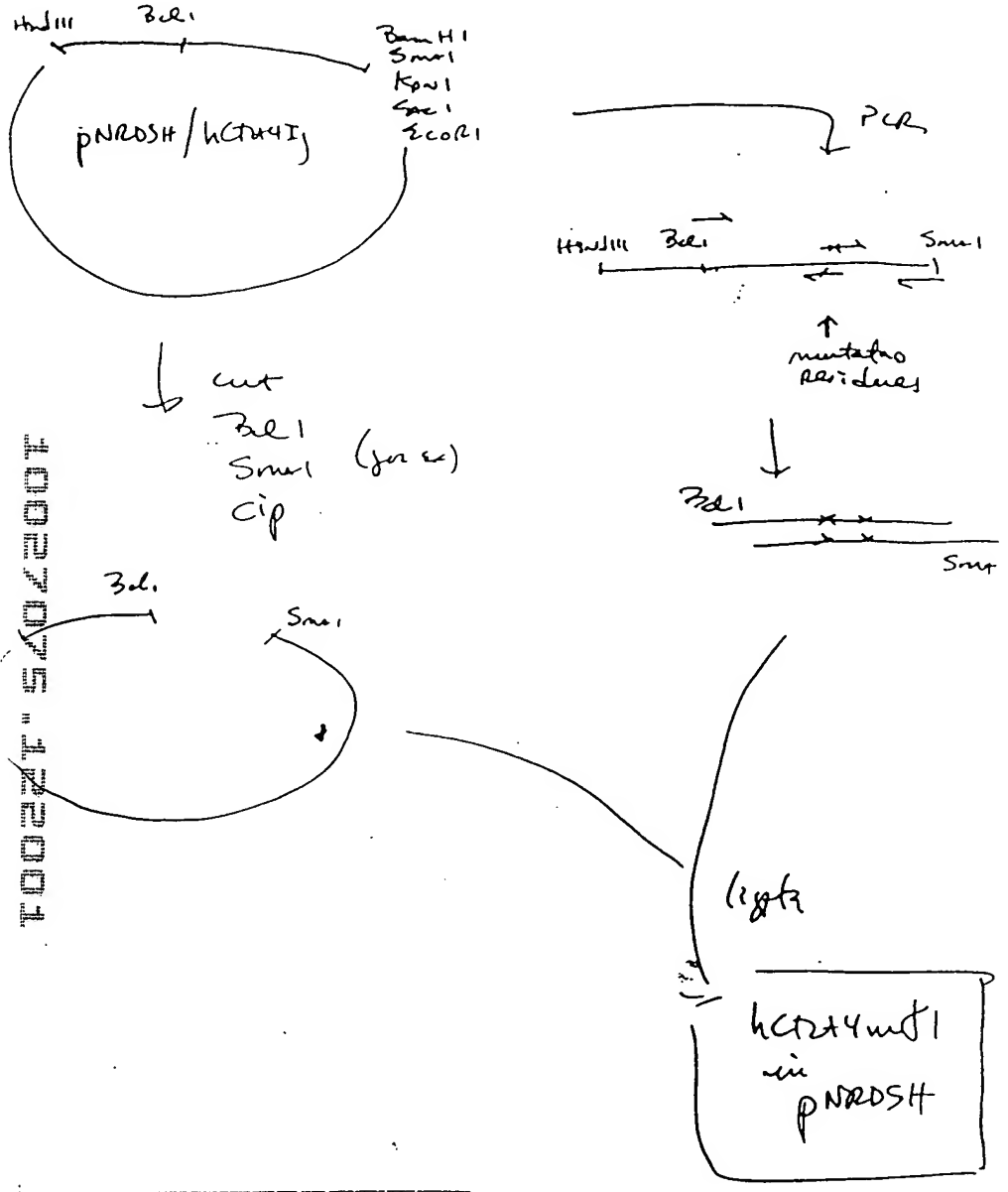
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

use NESTED PCR to generate a mutated 81 from hCTLA4Ij. Clone the mt81 back into hCTLA4 ___M = pNRDSH:



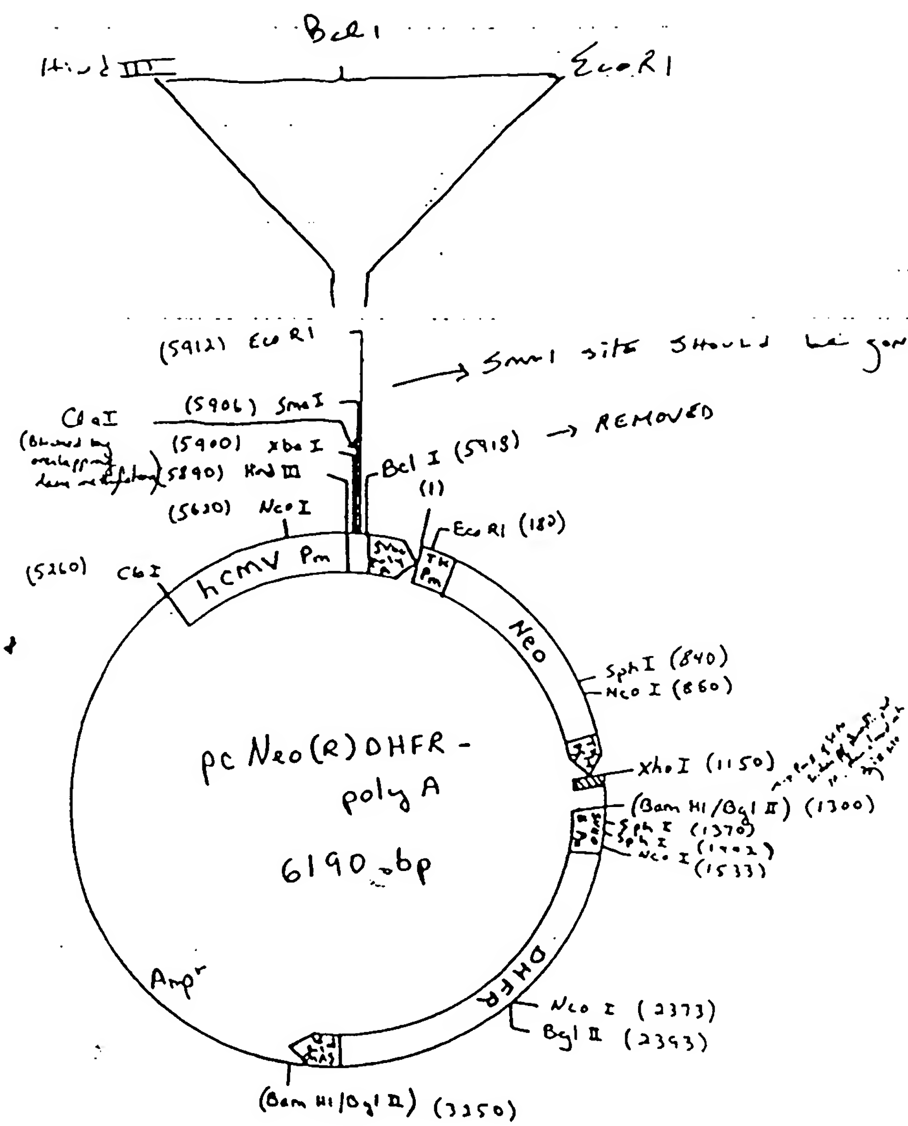
For this clone mutate residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3

Spe I 1227 p3

Kpn I (1-9-92)

Read and understood by me

Date

100221 "1520750"1001

primers for γFc mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q)

E S . K Y

BCL1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

sequence →

(G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

1st use this

3' primer

if NEEDED still have these →

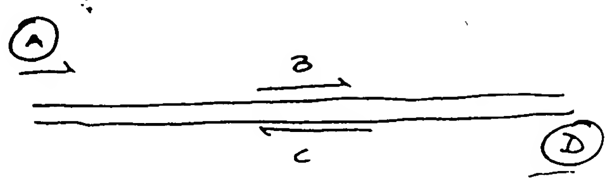
5' GCAGAG GAATTC GAGCTC GGTACCC GGGG ATCC

lock RI SacI KpnI XmaI BamHI

γ4 me CCAGTGTGGGG ACAGTGGG A CC CGCTCT GCCTCCC 3'

Read and understood by me [signature] Date

Fr-kg7



5' primer ✓

(A): use Gary Gray's original δ, primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT GAC AAA ACT
CAC ACA TCT CCA CCG TCT CCA GGT AAA C — IgFc —
—*— BamHI - SmaI - KpnI - SacI - EcoRI - ClaI - EcoRS - BglII —
— T7 promoter

3' primer (D):

pSP72 MCS: 5' GGATCCCC GGGTACC GAGCTC GAATTC 3' (BamHI, SmaI/XmaI, KpnI, SacI, EcoRI)
3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAGCTCGGGTACCCGGGGATCC
(lock)

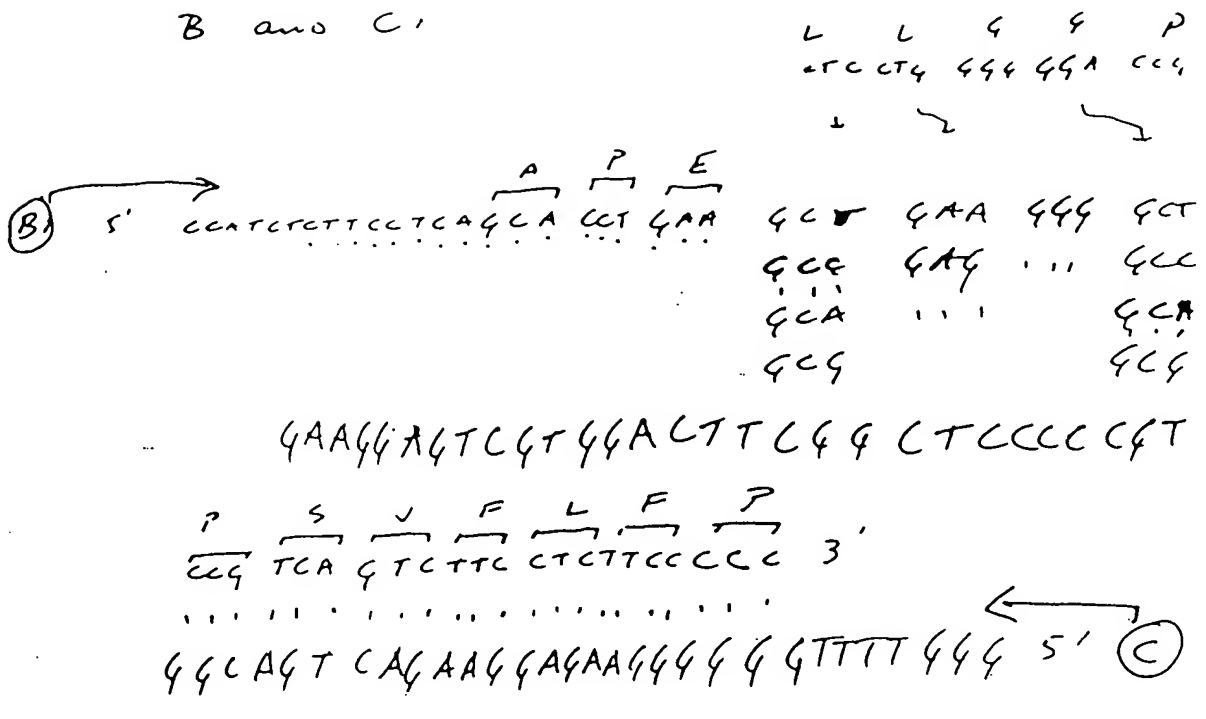
Read and understood by me

Date

1111

A-7

B and C'



Oligonucleotide Requests

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rownat

PROJECT CHARGED 87 & 90T

DATE REQUESTED .

DATE REQUIRED (NO ASAP)

SEQUENCE NAME Mugamma 4 - 5'

LENGTH 67

SEQUENCE:

5' GAGCATTTTCCTGATCAGGA
GTCCAAAATATGGTCCCCCAT
CCCCATCATCCCCAGGTAAG
CCAAACCC 3'

Read and understood by me

Date

A-8

Transient Expression of IgL hCTLA4(3) Ig / EF12

→ 3F

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

SAMPLE IDENTIFICATION	ug/mL IgG 1	ug/10^6/72hrs IgG 4	Dilutions 1:10 → 1:2
IgL CTLA4(+2)-γ1 1	2.12	1.77	↓
uncoCTLA4-mγ4 2	14.88	3.23	↓
IgL CTLA4(-2)-γ1 3	34.26	33.65	↓
IgL CTLA4(3)-γ1 4	33.9	35.54	↓

⊕ Controls — 2.25 μg/ml — 156 ng/ml

expected: — 2 μg/ml — 125 ng/ml

These samples were tested for their ability to compete for B7 bin. Assay run by Nancy Green.

Optical Density

	1 unlabelled CTLA4-Ig	2 #1 (JC samples)	3 #2	4 #3	5 #4	6 WT Avi 6/16	7 7/13	8 5T mpHL 7/13	9 Aug A1104	10 Dup TM6	11	12
70 μg/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
11.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
5.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max. signal – no inhibitor)

As before the IgL hCTLA4 is not functional. The two clones of IgL hCTLA4 do effectively compete w/ CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NSO lines.

→ Samples titrated serially 1:2 – in 50 μl

→ All sample wells contain 50 μl of 70 ng/ml CTLA4-Ig biotin

→ 43

Read and understood by me

Date

100225.20001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver Serial No.: ~~09/227,595~~ Continuation of 09/227,595 Filed: ~~January 8, 1999~~ December 20, 2001 For: CTLA4-Cγ4 Fusion Proteins (as amended) Attorney Docket No.: RPN-001	Group Art Unit: 1642 Examiner: Helms, L.

Assistant Commissioner for Patents
Washington, D.C. 20231

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December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor for Megan E. Williams
~~Megan E. Williams~~
Registration No. 43,270
Attorney for Applicants

Mailing Label No. EL 833315914US

MEW

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

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2. Prior to July 11, 1994, the invention described and claimed in the above-referenced application was completed in this country, as evidenced by the following:

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Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: _____ Signed: _____

Gary S. Gray

Date: October 4, 2001 Signed: _____

Jerry Carson

Date: _____ Signed: _____

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: _____ Signed: _____

Sandra Silver

human-CTLA4: Ig

STATUS: This clone, which is currently producing large amounts of active protein in CHO DG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 4
Jung et al, 1991 J Immunol. (147)
TAO et al, 1991 J Exp Med (173) 02
Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ1 and mutated residues 235 and 237 in γ4

See p 115, Book 1139 and

See p 52, Book 1177 and

Read and understood by me

Date

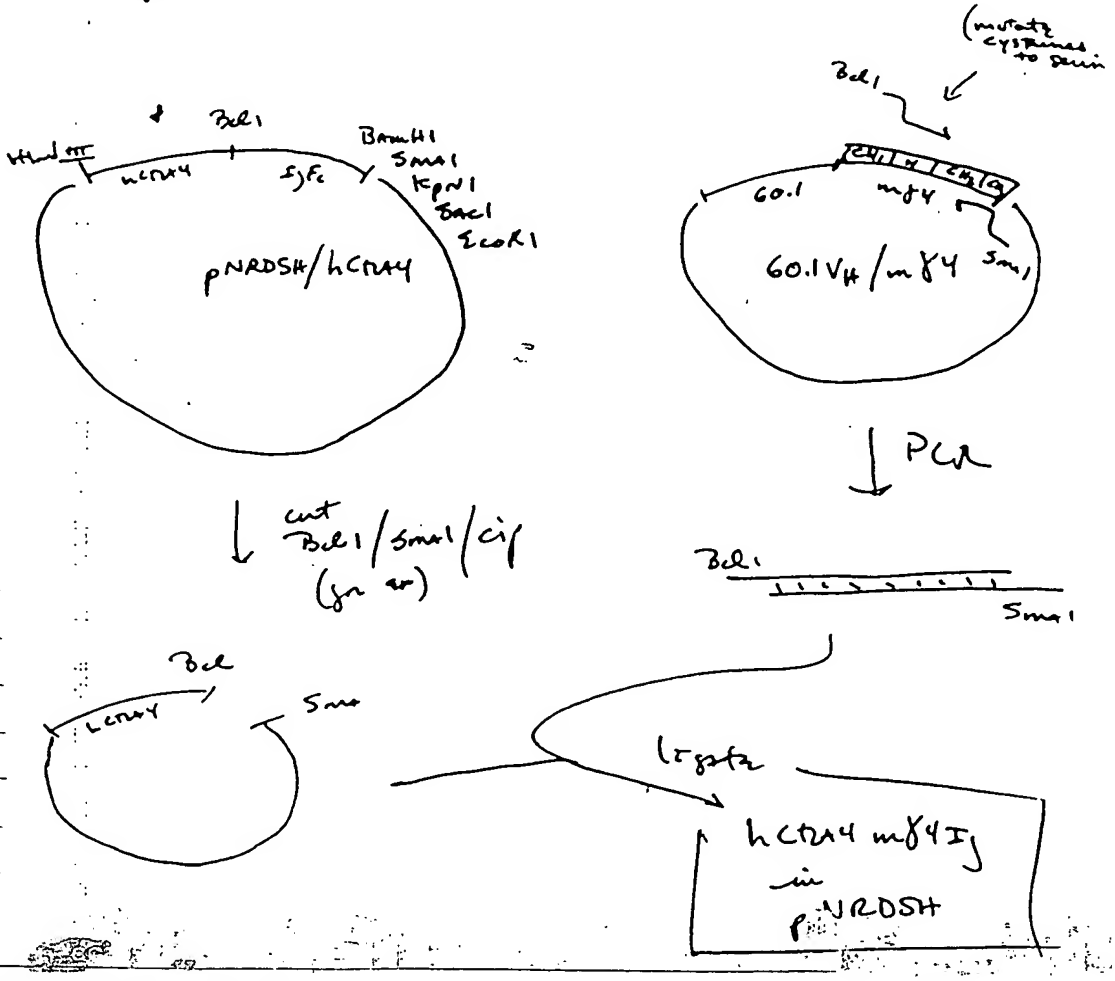
2 STRATEGIES will be USED:

hCγ4, mutagenesis of I_gFc

possible strategies:

① PCR out the mutated γ4 $H-CH_2-CH_3$ region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the existing γ_1 $H-CH_2-CH_3$

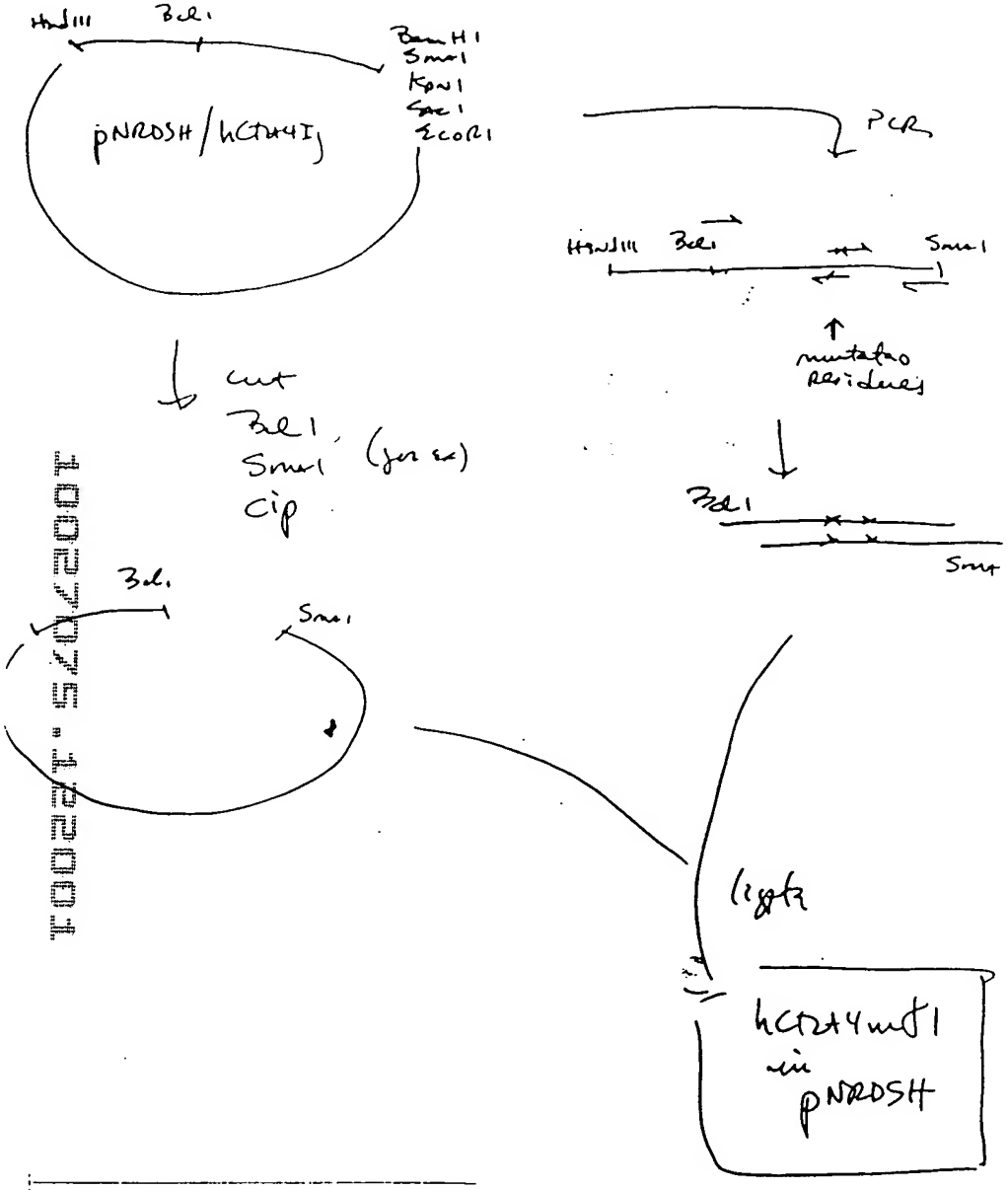
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

use NESTED PCR to generate a mutated 81 from hCTL4 Ij. Clone the mut 81 back into hCTL4. M pNRDSH:



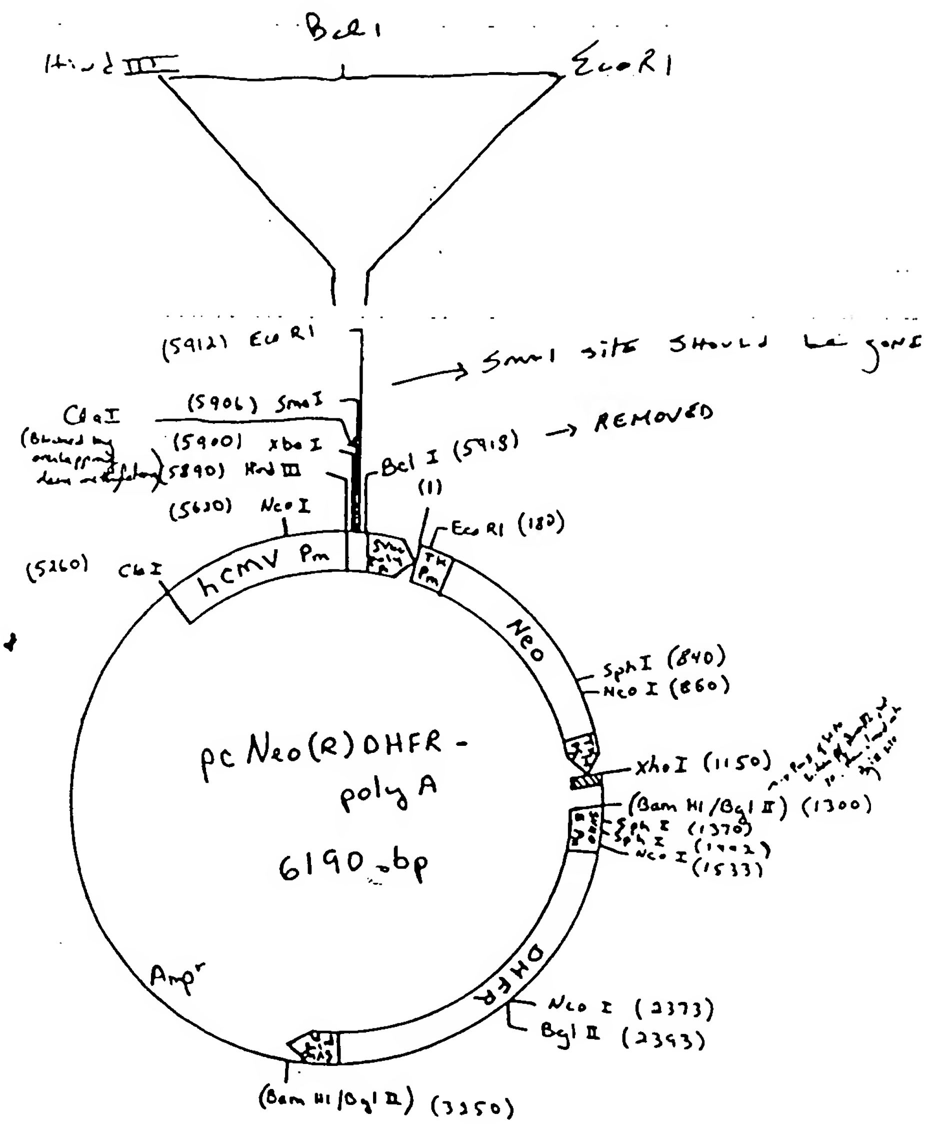
For this clone mutated residues as follows:

234	L → A
235	L → E
236	G
237	G → A

Read and understood by me

Date

Vector:



preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3

SpeI 1227 p3

KpnI (4-9-92)

→

Read and understood by me

Date

primers for γFc mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL 1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

sequence →

Ⓖ Ⓚ Ⓟ Ⓣ

GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

1st use this

3' primer

if NEEDED still have these → →

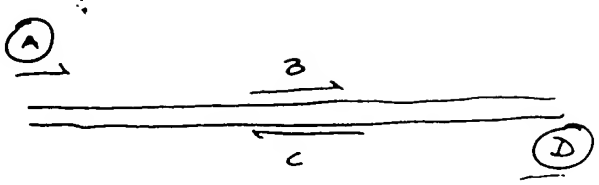
5' GCAGAG GAATTC GAGCTC GGTACCC GGGG ATCC

lock R1 SacI KpNI XmaI BamHI

[illegible] CCAGTGTGGGG ACAGTGGG A CC CGCTCT GCCTCCC 3'

Read and understood by me

Date



5' primer ✓

(A): use Gary Gray's original 5' primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CTC AAA TCT TCT CTC AAA ACT
P Bgl2 Q E P K S S D K T
CAC ACA TCT CCA CTG TCT CCA GGT AAG C — Pj Pc —
H T S P P S P G K

— * — BamHI - SmaI - KpnI - SacI - EcoRI - ClaI - EcoRS - Bgl2 —

— T7 promoter

3' primer (D): XmaI

BamHI SmaI KpnI SacI EcoRI

pBR322 MCS: 5' GGATCCCC GGGTACC GAGCTC GAATTC 3'

3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAGCTCGGTACCCGGGGATCC 3'

lock

Read and understood by me

Date

1111 A-7

B and C:

L L G G P
ATC CTG GGG GGA CCG

(B) 5' CCATCTCTTCCTCAGCA CCT GAA GCT GAA GGG GCT
GCG GAG ... GCC
GCA ... GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCC CGT

P S V F L F P
CCG TCA GTC TTC CTC TTC CCC C 3'
... ...
GGCAGT CAG AAG GAG AAG GGG G G TTTT GGG 5' (C)

Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Bennett

PROJECT CHARGED B7 & TGT

DATE REQUESTED .

DATE REQUIRED (NO ASAP)

SEQUENCE NAME mGAMMA 4 - 5'

LENGTH 67

SEQUENCE:

5' GAGCATTTTTCCTGATCAGGA
GTCCAAAATATGGTCCCCAT
CCCCATCATCCCCAGGTAAG
CCAAACCC 3'

Read and understood by me

Date

Transient Expression of IgLhcCTLA4 Ig / FEIL

A-8

→ 3F

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

IDENTIFICATION		ug/mL IgG1	IgG4	Dilutions 1:10 → 1:2
ILL CTLA4(+2)-γ1	1	2.12	1.77	↓
CTLA4-m γ4	2	14.88	3.23	
IgL CTLA4(+2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.9	35.54	

⊕ Controls 2.25 µg/ml 156 ng/ml

expected: 2 µg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 bin. Assay run by Nancy Green.

Optical Density

	1 (unlabelled CTLA4 Ig)	2 (JC samples #1)	3 (#2)	4 (#3)	5 (#4)	6 (WT Avi 6/16)	7 (7/13)	8 (WT rpt 7/13)	9 (Aug A504)	10 (rpt TM6)	11	12
50 ng/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
15.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(no unlabeled — signal — maximum)

As before the IgLLCTLA4 is not functional. The two clones of IgLhcCTLA4 do effectively compete w/ CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NSO lines.

→ Samples titrated serially 1:2 - in 50 µl vol

→ All sample wells contain 50 µl of 70 ng/ml CTLA4 Ig biotin

→ 43

Read and understood by me

Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver Continuation of NEW Serial No.: 09/227,595 Filed: ~~January 8, 1999~~ December 20, 2001 For: CTLA4-Cγ4 Fusion Proteins (as amended) Attorney Docket No.: RPN-001	Group Art Unit: 1642 Examiner: Helms, L.

Assistant Commissioner for Patents
Washington, D.C. 20231

NEW Certificate ~~of First Class Mailing~~ (37 CFR ~~1.8(a)~~ Under 1.10)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ "Express Mail" addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor
~~Megan E. Williams~~ Larry Taylor
~~Registration No. 43,270~~
~~Attorney for Applicants~~ NEW

Mailing Label No. EL833139141US

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

2. Prior to July 11, 1994, the invention described and claimed in the above-referenced application was completed in this country, as evidenced by the following:

A. Prior to July 11, 1994, we were in possession of a cloned CTLA4Ig molecule which was producing large amounts of active protein in CHO DG44 cells.

Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

Page A-2 of Appendix A describes amplification of the mutated γ4 Hinge-CH_2-CH_3 region and the cloning of the mutated γ4 into pNRDSH/hCTLA4 to replace the existing γ1 Hinge-CH_2-CH_3.

Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: _____ Signed: _____

Gary S. Gray

Date: _____ Signed: _____

Jerry Carson

Date: 10-3-01 Signed: Kashi Javaherian

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: _____ Signed: _____

Sandra Silver

HUMAN - CTLA4 : Ig

STATUS : This clone, which is currently producing large amounts of protein in CHODG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
Linsley et al, 1991 J Immunol. (147)
Linsley et al, 1991 J Exp Med (173) 721
Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated residues 235 and 237 in γ_4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me

Date

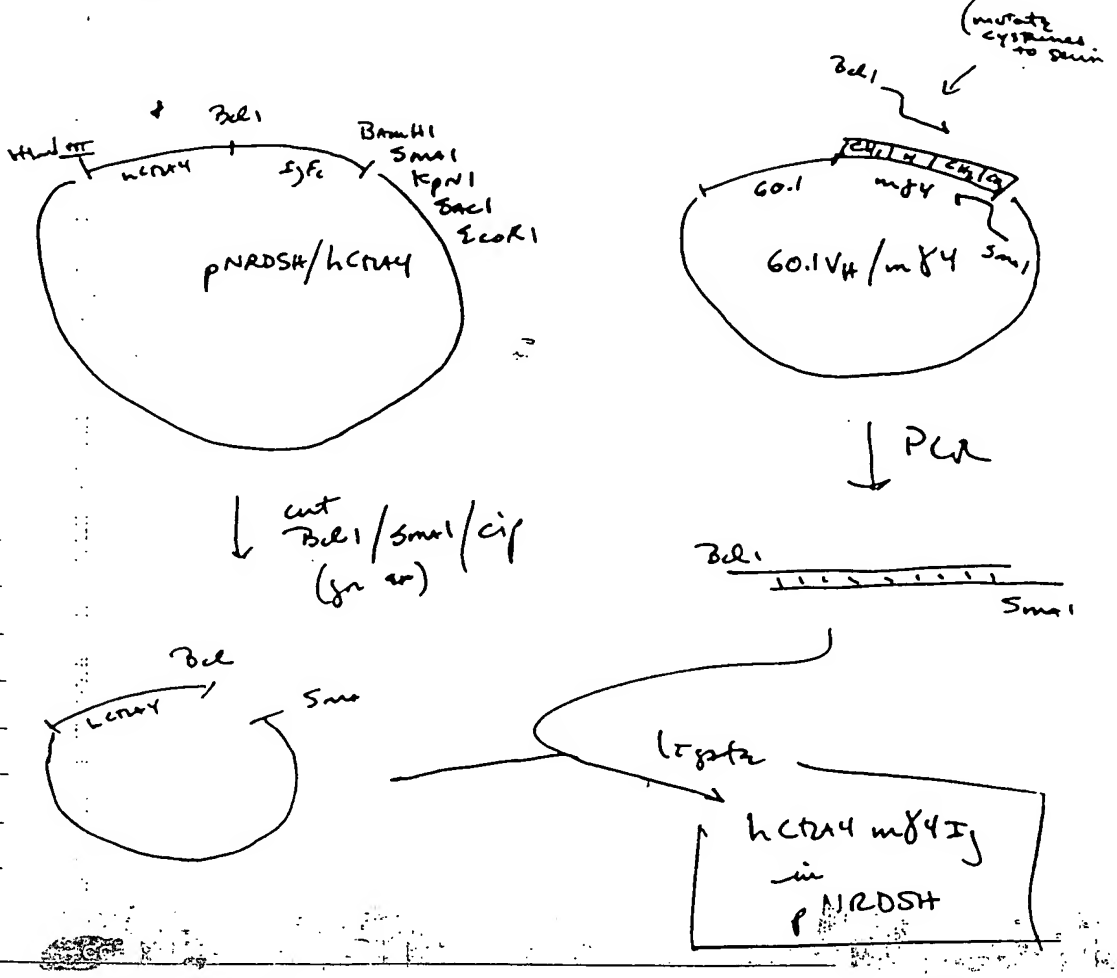
2 STRATEGIES will be USED:

hCγ4, mutagenesis of IgFc

possible strategies:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the existing γ_1 H-CH_2-CH_3

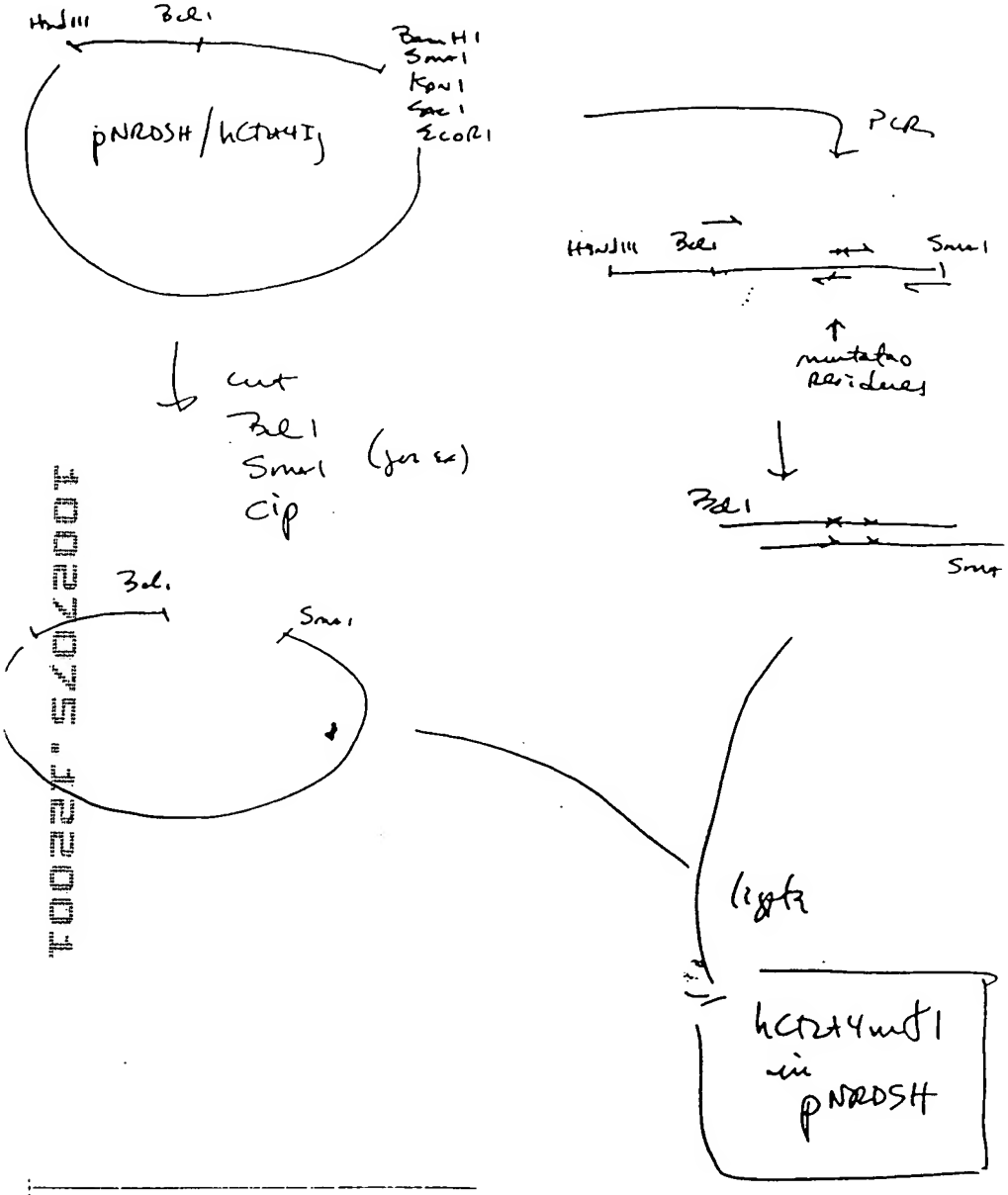
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

USE NESTED PCR to generate a mutated β1 from hCTLH4Ij. Clone the mut β1 back into hCTLH4. M pNRDSH:



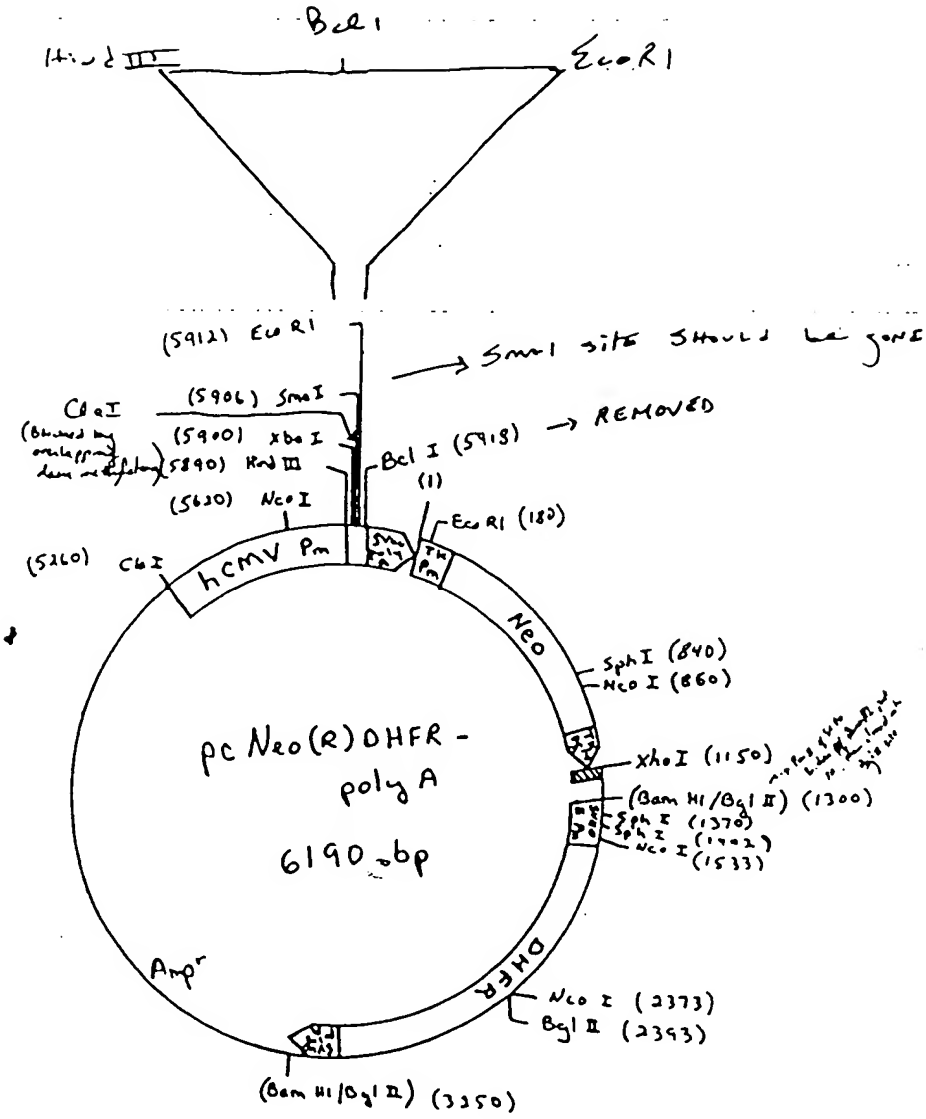
FOR this CLONE mutate residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (1-12)

Read and understood by me

Date

primers for γ4 mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL 1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

primer → (G) (K) (P) (T)

γ4 GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

3' primer

1st use this

if necessary still have these →

5' GCAGAG GAATTC GAG CTC GGTACCC GGGGATCC

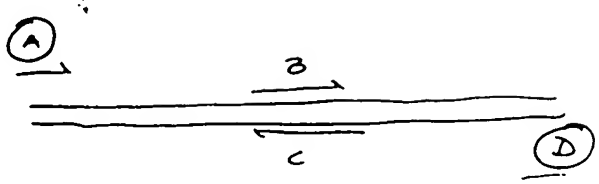
lock R1 Sac1 KpN1 Xma1 BamH1

γ4 mc CCAGTGTGGGG ACAG TGGG A CC CGCTCT GCC TCCC 3'

Read and understood by me

Date

Brad R Cou



5' primer ✓

(A): use Gary Gray's original 5' primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT
P Bgl2 Q E Q K S S D K T
CAC ACA TCT CCA CCG TCT CCA GGT AAG C — IgFc —
H T S P P S P G K

— * — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-Bgl2 —

— T7 promoter

3' primer (D): XmaI

BamHI SmaI KpnI SacI EcoRI

pSB22 MCS: 5' GGATCCCC GGGTACC GAGCTC GAATTC 3'

3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAG CTCGGTACCCGGGGATCC

lock

Read and understood by me

Date

A-7

B and C

L L G G P
cTC CTG GGG GGA CCG

A P E
(B) 5' CCATCTCTTCCTCAGCA CCT GAA GCT GAA GGG GCT
GCG GAG ... GCC
GCA ... GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCCCGT

P S V F L F P
CCG TCA GTCTTC CTCTTCCCCC 3'

GGCAGT CAGAAGGAGAAGGGGGGTTTT GGG 5' (C)

Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rennert

PROJECT CHARGED 37 # 9CT

DATE REQUESTED

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGAMMA4 - 5'

LENGTH 67

SEQUENCE:

5' GAGCATTTTCCTGATCAGGA (BclI)
GTCCAAAATATGGTCCCCCAT
CCCCATCATCCCAGGTAAG
CCAAGCC 3'

Read and understood by me

Date

A-8

Transient Expression of IgLhCTLA4 Ig / IEE12

→ 2F

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

SAMPLE IDENTIFICATION		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4(+2)-γ1	1	2.12	1.77	↓
onco CTLA4-mγ4	2	14.88	3.23	
IgL CTLA4(-2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.9	35.54	

⊕ Controls 2.25 μg/ml 156 ng/ml

expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 binding. Assay run by Nancy Ghan.

Optical Density

		1 (unlabelled CTLA4 Ig)	2 (JC samples #1)	3 (#2)	4 (#3)	5 (#4)	6 (WT)	7	8	9	10	11	12
70 ug/ml	A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25	B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5	C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25	D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
5.6	E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8	F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9	G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0	H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max signal — no inhibitor)

→ Samples titrated serially 1:2 - in 50 ul

→ All sample wells contain 50 of 70 ng/ml CTLA4 Ig biotin

As before the IgLhCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete w/ CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NS0 lines.

→ 43

Read and understood by me

Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver Serial No.: 09/227,595 Continuation of Filed: ~~January 8, 1999~~ December 20, 2001 For: CTLA4-Cγ4 Fusion Proteins (as amended) Attorney Docket No.: RPN-001	Group Art Unit: 1642 Examiner: Helms, L.

new

Assistant Commissioner for Patents
Washington, D.C. 20231

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December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor
~~Megan E. Williams~~
~~Registration No. 43,270~~
~~Attorney for Applicants~~ new

Mailing Label No. EL 933159 14 US

DECLARATION UNDER 37 C.F.R. 1.131 BY GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

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Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

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The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
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These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

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Date: ____________________ Signed: ________________________

Gary S. Gray

Date: ____________________ Signed: ________________________

Jerry Carson

Date: ____________________ Signed: ________________________

Kashi Javaherian

Date: 3 October 2001 Signed: Paul D Rennert

Paul D. Rennert

Date: ____________________ Signed: ________________________

Sandra Silver

HUMAN - CD44 : Is

STATUS : This clone, which is currently producing large amounts of soluble protein in CHO DG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
Jung et al, 1991 J Immunol. (147)
Tao et al, 1991 J Exp Med (173) 1025
Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated Residues 235 and 237 in γ_4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me

Date

[redacted]

[redacted]

[redacted]

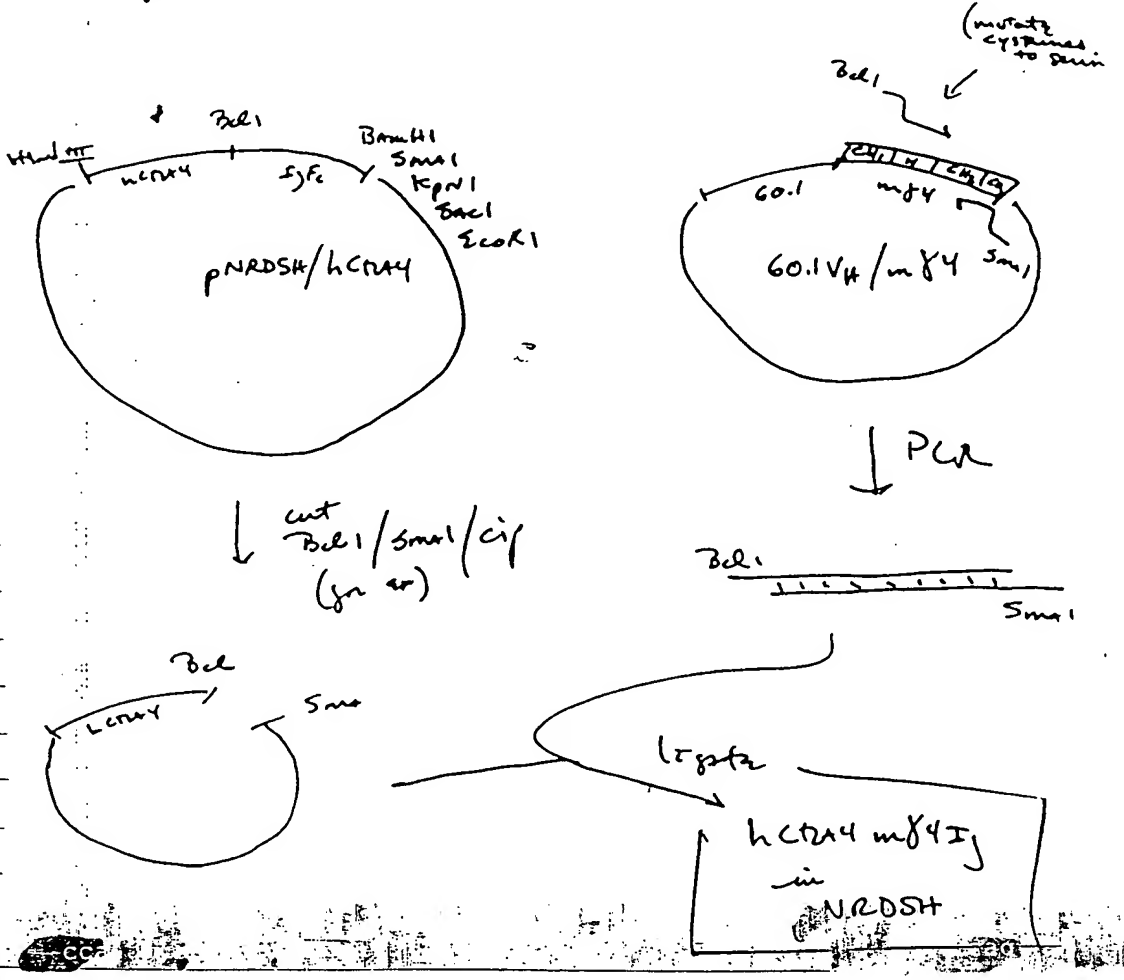
2 STRATEGIES will be USED:

hCγ4, mutagenesis of I_gFc

possible strategies:

① PCR out the mutated γ4 $H-CH_2-CH_3$ region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the EXISTING γ_1 $H-CH_2-CH_3$

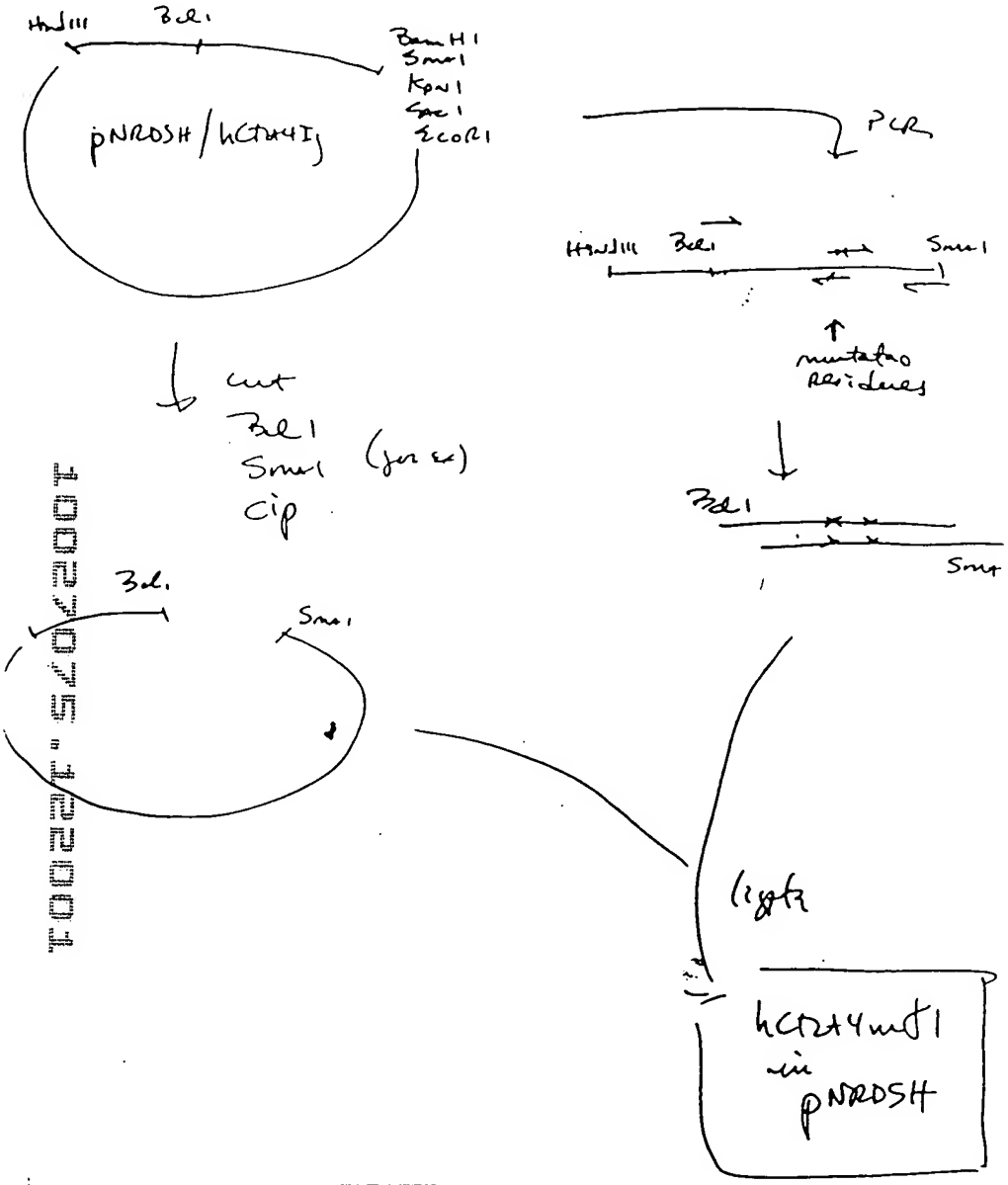
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

use NESTED PCR to generate a mutated 81 from hCTLA4Ij. Clone the mf1 back into hCTLA4 M pNRDSH:



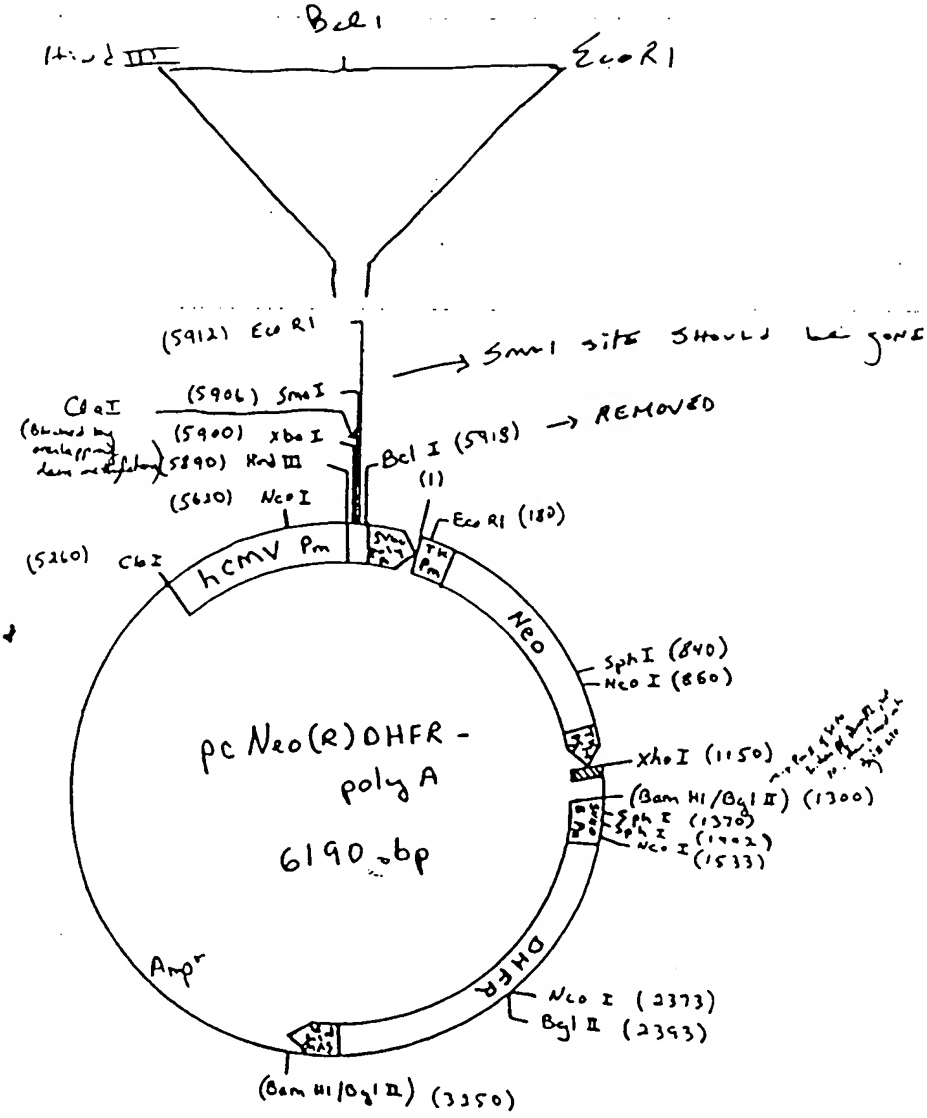
For this clone mutated residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



Enzymes that DO NOT CUT

EcoRV 1227 r3
SpeI 1227 r3
KpnI (1-9-92)

Read and understood by me

Date

primers for [redacted] mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL 1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

primer → (G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT PNRDSH LACKS THIS restriction site

1st use this

3' primer

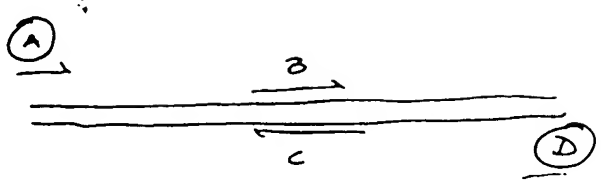
if needed still have these →

5' GCAGAG GAATTC GAG CTC GGTACCC GGGGATCC

lock R1 Sac1 KpN1 Xma1 BamH1

4 me CCAGTGTGGGG ACAG TGGG ACC CGCTCT GCC TCCC 3'

Read and understood by me [redacted] Date [redacted]



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT
P BglII Q E P K S S D K T
CAC ACA TCT CCA CCG TCT CCA GGT AAG C — $Ig\gamma_1$ Fc —
H T S P D S P G K

— * — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-BglII —

— T7 promoter

3' primer (D): XmaI

pBR322 MCS: BamHI SmaI KpnI SacI EcoRI

5' GGATCCCC GGGTACC GAGCTC GAATTC 3'

3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAGCTCGGTACCCGGGGATCC 3'

lock

Read and understood by me

Date

111 A-7

B and C:

L L G G P
CTC CTG GGG GGA CCG

(B) 5' CCATCTCTTCCTCAGCA CCT GAA GCT GAA GGG GCT
A P E
GCG GAG ... GCC
GCA ... GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCCCGT

P S V F L F P
CCG TCA GTC TTC CTCTTCCCCC 3'

GGCAGT CAGAAGGAGAAGGGGG GTTTT GGG 5' (C)

Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rennert

PROJECT CHARGED 37 # 1GT

DATE REQUESTED

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGamma4 -5'

LENGTH 67

SEQUENCE:

5' GAGCATTTTCCTGATCAGGA
GTCCAAATATGGTCCCCCAT
CCCCATCATCCCCAGGTAAG
CCAACC 3'

Read and understood by me

Date

A-8

Transient Expression of IgL hCTLA4(3) Ig / IFE12

→ 3F

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

CELL IDENTIFICATION		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4(+2)-γ1	1	2.12	1.77	↓
CTLA4-m84	2	14.88	3.23	
IgL CTLA4(+2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.9	35.54	

⊕ Controls 2.25 μg/ml 156 ng/ml

expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 binding. Assay run by Nancy Green.

Optical Density

		1 unlabelled CTLA4Ig	2 #1 (IC samples)	3 #2	4 #3	5 #4	6 WT	7	8	9	10	11	12
50 μg/ml	A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25	B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5	C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25	D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
5.6	E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8	F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
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0	H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max signal)

As before the IgL CTLA4 is not functional. The two clones of IgL hCTLA4 do effectively compete w CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NSO lines.

→ Samples titrated serially 1:2 – in 50 μl

→ All sample wells contain 50 of 70 ng/ml CTLA4Ig biotin

→ 43

Read and understood by me

Date

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Assistant Commissioner for Patents
Washington, D.C. 20231

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Mailing Label No. EL833315914US

By: Larry Taylor
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~~Attorney for Applicants~~

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Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: _____ Signed: _____

Gary S. Gray

Date: _____ Signed: _____

Jerry Carson

Date: _____ Signed: _____

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: October 16, 2001 Signed: Sandra Silver

Sandra Silver

HUMAN - CD44 : Ig

STATUS : This clone, which is currently producing large amounts of protein in CHO DG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
Jung et al, 1991 J Immunol. (147)
Tao et al, 1991 J Exp Med (173) 1025
Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated residues 235 and 237 in γ_4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me

Date

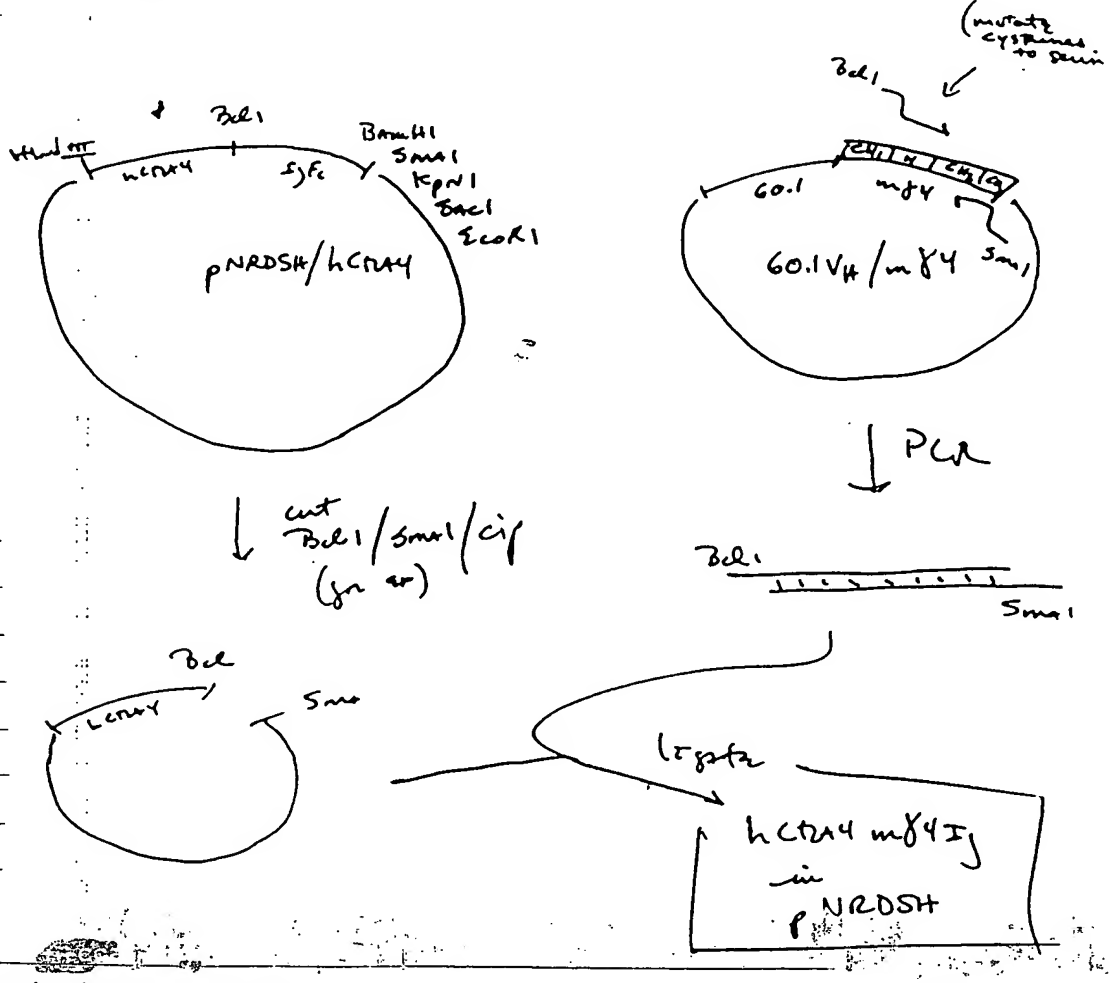
2 STRATEGIES will be USED:

hCH1-4, mutagenesis of IgFc

possible strategies:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCH1-4 in place of the existing γ_1 H-CH_2-CH_3

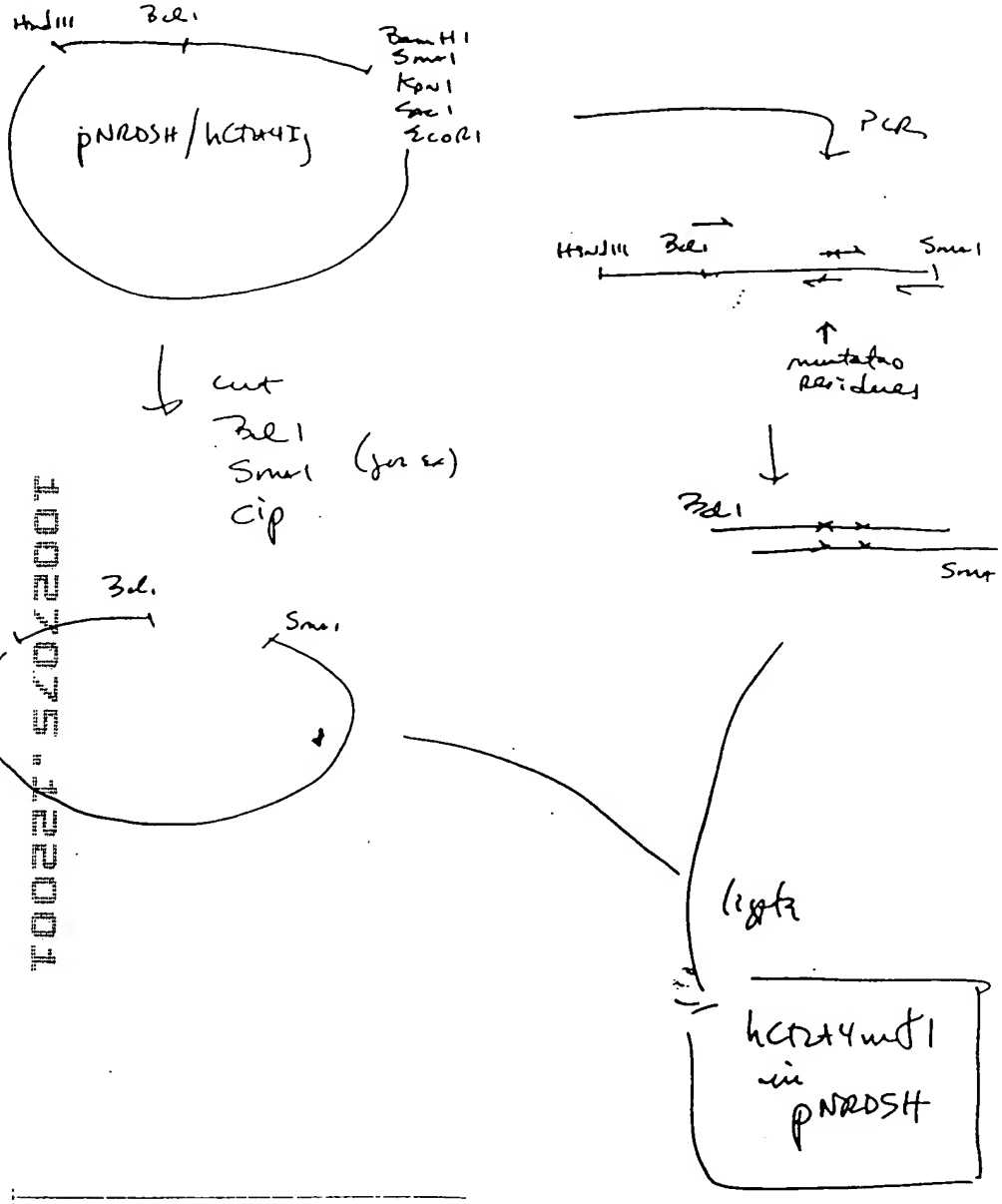
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

use NESTED PCR to generate a mutated δ1 from [redacted] hCTLA4Ij. Clone the mδ1 back into hCTLA4 [redacted] M pNRDSH:



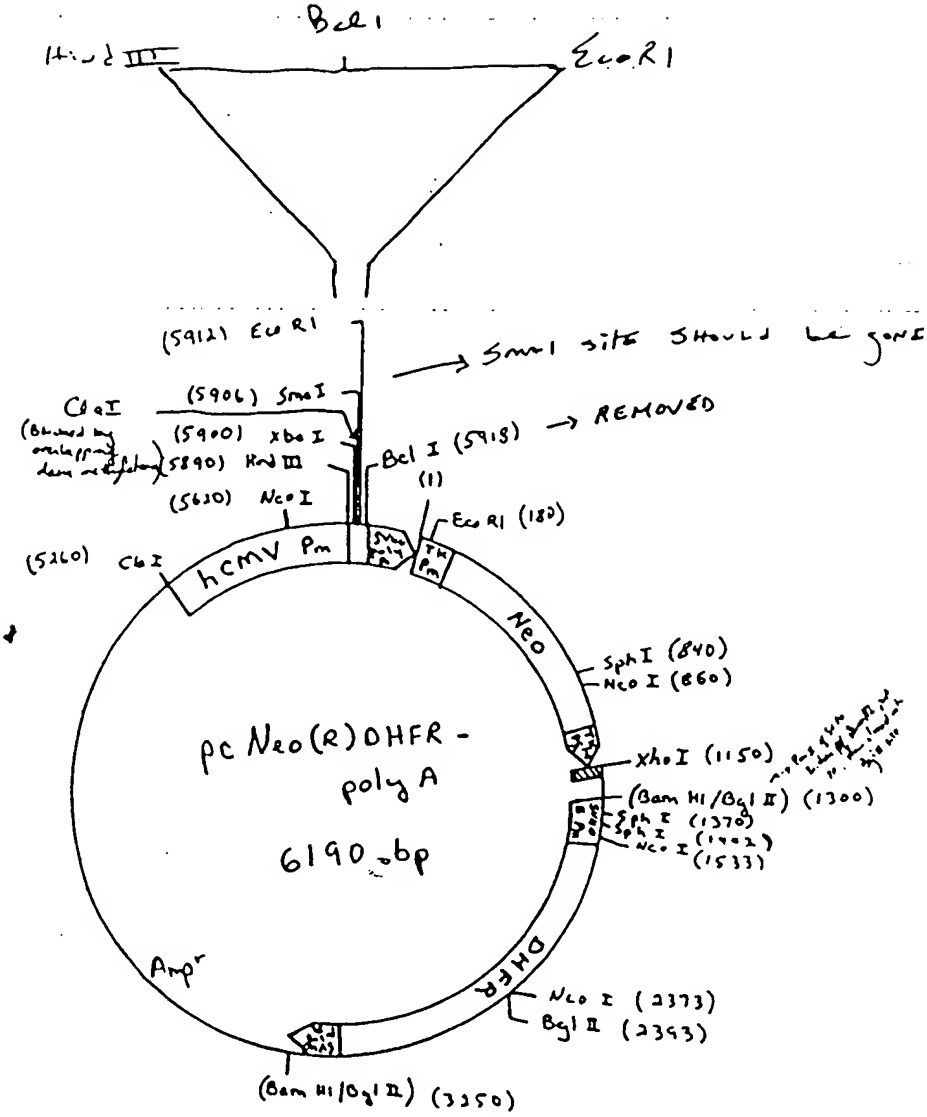
For this clone mutated residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (4-9-92)

Read and understood by me

Date

primers for [redacted] mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

(G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT PNRDSH LACKS THIS restriction site

1st use this

3' primer

if needed still have these →

5' GCAGAG GAATTC GAG CTC GGTACCC GGGGATCC

lock R1 Sac1 KpN1 Xma1 BamH1

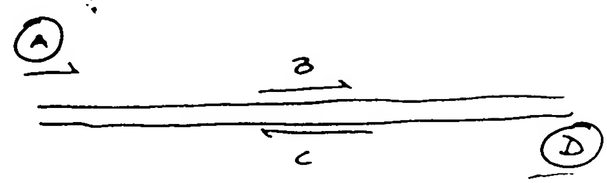
CCAGTGTGGGG ACAG TGGG A CC CGCTCT GCC TCCC 3'

Read and understood by me

Date

[redacted]

A-6



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT GAC AAA ACT
(P, D BcL1, Q, E, P, K, S, S, D, K, T)

CAC ACA TCT CCA CCG TCT CCA GGT AAA C — IgFc —
(H, T, S, P, P, S, P, G, K)

—— * —— BamHI - SmaI - KpnI - SacI - EcoRI - ClaI - EcoRV - BglII —

— T7 promoter

3' primer (D):

pSP72 MCS:

(BamHI, SmaI, XmaI, KpnI, SacI, EcoRI)

5' GGATCCCC GGG TACC GAGCTC GAATTC 3'
3' CCTAGGGG CCC ATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAGCTCGGTACCCGGGGATCC

(lock)

Read and understood by me

Date

10027075.122001

/// A-7

B and C:

L L G G P
CTC CTG GGG GGA CCG

(B) 5' CCATCTCTTCCTCAGCA CCT GAA GCT GAA GGG GCT
A P E
GCC GAG ... GCC
GCA ... GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCCCGT

P S V F L F P
CCG TCA GTCTTC CTCTTCCCCC 3'

GGCAGT CAGAAGGAGAAGGGG GGTTTT GGG 5' (C)

1000270'5'1E20001

Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rennert

PROJECT CHARGED 37 # 1GT

DATE REQUESTED

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGAMMA4 - 5'

LENGTH 67

SEQUENCE:

5' GAGCATTTTCCTGATCAGGA (BclI)

GTCCAAAATATGGTCCCCCAT

CCCCATCATCCCAGGTAAG

CCAACCC 3'

Read and understood by me: [signature]

Date

A-8

Transient Expression of IgL hCTLA4 - Ig / E212

→ 38

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

Cell Identification		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4 (+2) - γ1	1	2.12	1.77	↓
onco CTLA4 - mγ4	2	14.88	3.23	
IgL CTLA4 (-2) - γ1	3	34.26	33.65	
IgL CTLA4 (3) - γ1	4	33.9	35.54	

⊕ Controls 2.25 µg/ml 156 ng/ml

expected: 2 µg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 binding. Assay run by Nancy Green.

Optical Density

	1 unlabeled CTLA4 Ig	2 JC samples #1	3 #2	4 #3	5 #4	6 WT	7	8	9	10	11	12
70 µg/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
15.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(no competing signal)

As before the IgLhCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete w CTLA4-Ig -biotinylated.

Plasmids are ready for transfection into stable NSO lines.

→ Samples titrated serially 1:2 - in 50 µL

→ All sample wells contain 50 of 70 ng/ml CTLA4 Ig biotin

→ 43

Read and understood by me

Date